Baseless Assumptions: Activation of TLR9 by DNA

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The Toll-like receptor 9 (TLR9) is activated by DNA presented in acidified, intracellular compartments. Previous studies suggested that signaling required unmethylated CpG dinucleotides, but in this issue of *Immunity*, Haas et al. (2008) challenge this view, showing that DNA can activate TLR9 in a sequence-independent manner.

One of the most surprising discoveries to arise from research on pathogen pattern recognition is the finding that a subgroup of the Toll-like receptors (TLRs) signal in response to nucleic acids and small immunostimulatory drug molecules such as the imidazoquinolines (Akira et al., 2006). More specifically, TLR9 was found to respond to DNA oligonucleotides bearing unmethylated CpG base pairs, TLR7, and TLR8 to single-stranded RNA and TLR3 to double-stranded RNAs (dsRNAs). An important requirement of these recognition processes is that they must be able to distinguish pathogen-associated and self nucleic acids perfectly in order to prevent the generation of dangerous autoimmune responses (Barton et al., 2006). It was thought that this discrimination might act at two levels. First, the receptors would recognize specific structural features associated with nonself nucleic acids such as the lack of cytosine methylation in many bacterial and viral genomes. Second, unlike other TLRs, the TLR3, TLR7, TLR8, and TLR9 subgroup are localized to intracellular compartments such as the endosome and lysosome and require an acidic pH to function. Thus, to stimulate an innate immune response the nonself nucleic acid must be both structurally distinct and presented to the receptors in specific intracellular compartments.

In this issue of *Immunity*, Haas et al. (2008) challenge this view of innate immune recognition of nucleic acid, showing that activation of TLR9 does not require nonself CpG containing DNA. Instead, they show that TLR9 can recognize DNA independently of base sequence and that the 2′ deoxyribose is sufficient to confer signaling specificity by TLR9 for DNA as opposed to RNA. Previous studies defined the CpG sequence specificity with nonnatural phosphorothionate (PS) oligonucleotides in which one of the oxygen atoms of the phosphate backbone is substituted for sulfur (Figure 1). The rationale for this was that PS oligonucleotides are protected from degradation by nucleases and can more easily be delivered to the intracellular compartments from which TLR9 signals. Haas et al. set out to discover whether natural phosphodiester (PD) containing single-stranded DNA molecules had the same sequence requirements as the PS derivatives (Figure 1). To do this, they stabilized the PD oligonucleotides by adding poly-G sequences. Contrary to expectation, deoxy PD molecules but not the corresponding RNA ribo-oligonucleotides could activate TLR9 irrespective of their base sequence. Furthermore, polymers of 2′ deoxyribose lacking any bases could also activate TLR9 and bind to the extracellular, ligand-recognition domain (ectodomain) of TLR9 in vitro. They also tested the binding of PS and PD oligonucleotides to the ectodomains of TLR9 and the RNA-activated TLR7. Deoxy PS and PD polymers bound to TLR9, but ribo-oligonucleotides interacted only with TLR7. These results emphasize the importance of the 2′deoxy group and 2′ ribo groups for specific recognition of DNA and RNA by TLR9 and TLR7, respectively. Finally the authors show that, like PS oligonucleotides lacking CpG sequences, PS versions of base-free 2′ deoxyribose also inhibit TLR9 signaling. Haas et al. conclude that unmethylated CpG DNA only activates TLR9 in the context of the nonnatural PS backbone. The reasons for this are not clear but presumably reflect chemical differences in PS oligonucleotides conferred by the phosphorothionate group (Figure 1).

An important question raised by this study is how TLRs are able to discriminate between DNA and RNA molecules and what the basis of pH-dependent activation of the nucleic acid TLR subgroup is. In the case of TLR3, which responds to double-stranded RNA but not to DNA, residues that are required for this specificity have been identified in the ectodomain (Ranjith-Kumar et al., 2007). These residues are mainly amides, asparagine and glutamine, and the side chains may make specific hydrogen bonds to the 2′ –OH of the ribose (Figure 1). These studies also identified a histidine residue that is involved in dsRNA binding. This is important because the imidazole group of histidine becomes positively charged when the pH is lowered from 7 to 5 and could mediate the pH-dependent activation observed for these TLRs. By using a sequence alignment, Haas et al. identify the corresponding residues in TLR9. They find that the histidine residue is not conserved but is replaced by an uncharged glutamine. They propose that this residue may hydrogen bond to 2′deoxyribose in DNA an interaction that could also be made with RNA (Figure 1). In contrast, a second critical residue in TLR3, asparagine 541, is replaced by arginine in TLR9 and may confer specificity for DNA. For steric reasons, the bulky arginine side chain could make stabilizing interactions with the 2′ deoxyribose (a very weak hydrogen-bond donor) but not to the 2′ OH of RNA (Figure 1). Interestingly, a large number of contacts with the sugar group of DNA seen in known structures are mediated by arginine (Luscombe et al., 2001). Although these findings provide interesting clues about the nature of nucleic-acid recognition by the TLRs, many questions remain unanswered. For example, if histidine side chains in the ectodomain are not required for pH-dependent binding and signaling, could it be that there

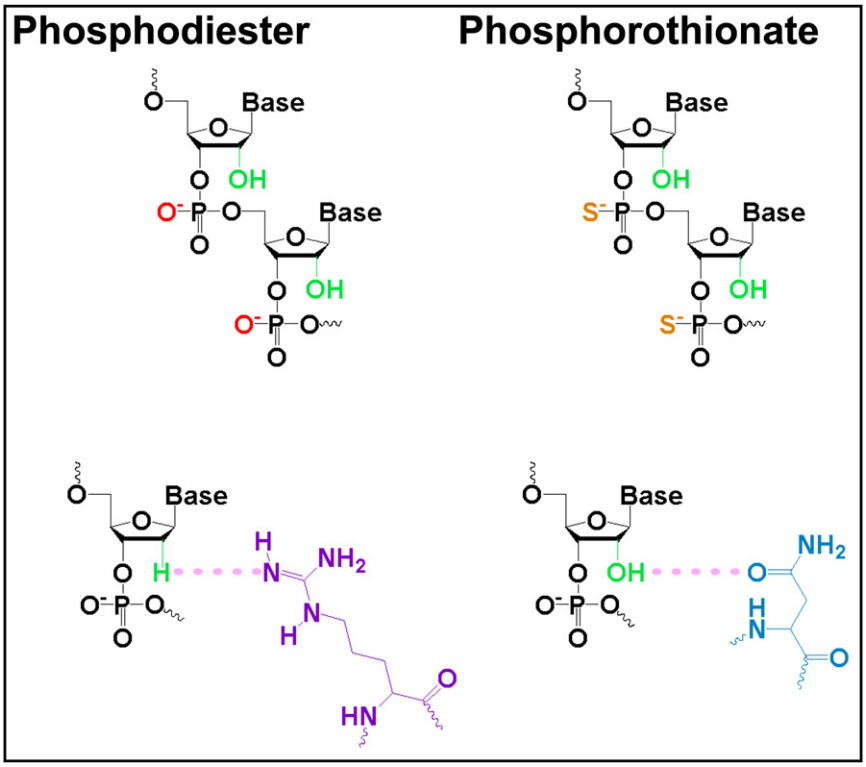

Figure 1. Recognition of DNA by TLR9
Structure of phosphodiester (PD) and phosphorothionate (PS) oligonucleotides. The 2′ position is shown in green and is –OH in RNA and –H in DNA. The oxygen atom of the phosphate backbone shown in red is replaced by a sulfur atom in phosphorothioate (PS) oligonucleotides. Also shown is the potential noncovalent interactions between TLR9 and PD oligonucleotides that may be specific for DNA and RNA; hydrogen bond from an arginine side chain to the 2′ deoxy C-H group of DNA, and hydrogen bond from the amide side chain of asaparagine or glutamine to the 2′ OH of RNA.

are pH-dependent changes in the nucleic-acid ligands? If this is the case, then it is likely to involve the bases, particularly cytosine, because these are the only groups with pK_a values in the range of 4–8 whose ionization state is likely to change substantially over the pH range 5–7.

The study also raises intriguing questions about how nucleic-acid binding is coupled to receptor activation. The non-CpG PS oligonucleotides bind 100 times more strongly than corresponding PD molecules to the TLR9 ectodomain but do not activate signaling. Furthermore, baseless PS but not PD deoxyribose is a competitive inhibitor of TLR9, suggesting that both PS and PD DNA bind to the same sites. How then is binding coupled to activation? A recent study reveals that dsRNA binds to TLR3 and that binding indirectly induces dimerization of two TLR3 ectodomains (Leonard et al., 2008). Association of dsRNA with TLR3 also displays cooperativity, indicating that initial ligand binding induces conformational changes that are in the receptor and that drive the process of receptor activation (see [Gay et al., 2006]). The inhibitory properties of PS oligonucleotide may reflect an inability of this nonnatural molecule to promote the cooperative conformational changes required to initiate signal transduction. Structural studies for the TLR1-TLR2 complex with triacylated lipid (Jin et al., 2007) and *Drosophila* Toll with the protein ligand Spatzle show that in both cases, binding of ligand induces protein-protein contacts between the lateral surfaces of two TLR ectodomain molecules. This in turn causes the C-terminal juxtamembrane sequences of the two receptor molecules to be in close proximity. It may be that this is a common theme in TLR signaling shared by the nucleic-acid-activated subgroup. Indeed, fluorescence techniques detect cooperative changes in full-length TLR9 when it is activated by PS CpG DNA (Latz et al., 2007).

Many of the questions raised will be resolved by further studies of TLR9 in complex with activating and inhibiting DNA. In particular, high-resolution structural studies will reveal the precise nature of the protein DNA contacts with both PS and PD DNA molecules and the conformational changes that may accompany receptor activation.

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